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High glucose induces MCP-1 expression partly via tyrosine kinase–AP-1 pathway in peritoneal mesothelial cells

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High glucose induces MCP-1 expression partly via tyrosine kinase–AP-1 pathway in peritoneal mesothelial cells.

Background. High glucose in peritoneal dialysis solutions has been implicated in the pathogenesis of peritoneal fibrosis in chronic ambulatory peritoneal dialysis (CAPD) patients. However, the mechanisms are not very clear. Peritoneal macrophages seem to participate in the process of peritoneal fibrosis and monocyte chemoattractant protein-1 (MCP-1) plays a key role in the recruitment of monocytes toward the peritoneal cavity. However, little is known about the effect of high glucose on MCP-1 expression and its signal transduction pathway in human peritoneal mesothelial cells.

Methods. Mesothelial cells were cultured with glucose (5 to 100 mmol/L) or mannitol chronically for up to seven days. MCP-1 expression of mRNA and protein was measured by Northern blot analysis and enzyme-linked immunosorbent assay (ELISA). Chemotactic activity of high-glucose–conditioned culture supernatant was measured by chemotactic assay. To examine the roles of the transcription factors activator protein-1 (AP-1) and nuclear factor-κB (NF-κB), electrophoretic mobility shift assay (EMSA) was performed.

Results. Glucose induced MCP-1 mRNA expression in a time- and dose-dependent manner. MCP-1 protein in cell culture supernant was also increased. Equivalent concentrations of mannitol had no significant effect. High-glucose–conditioned supernatant possessed an increased chemotactic activity for monocytes, which was neutralized by anti–MCP-1 antibody. EMSA revealed that glucose increased the AP-1 binding activity in a time- and dose-dependent manner, but not NF-κB. Curcumin, an inhibitor of AP-1, dose-dependently suppressed the induction of MCP-1 mRNA by high glucose. Tyrosine kinase inhibitors such as genistein (12.5 to 50 μmol/L) and herbimycin A (0.1 to 1 μmol/L) inhibited the high-glucose–induced MCP-1 mRNA expression in a dose-dependent manner, and also suppressed the high-glucose–induced AP-1 binding activity.

Conclusions. High glucose induced mesothelial MCP-1 expression partly via the tyrosine kinase-AP-1 pathway.

Key words: CAPD, dialysate, fibrosis, monocyte chemoattractant protein-1, activator protein-1, signal transduction.

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Conventional peritoneal dialysis solutions used for continuous ambulatory peritoneal dialysis (CAPD) patients are characterized by unphysiologically high glucose concentration as well as high osmolality and low pH, etc. [1]. High glucose in peritoneal dialysis solutions has been implicated in the pathogenesis of peritoneal fibrosis, which is one of the serious long-term complications. High glucose may modulate the metabolism of peritoneal mesothelial cells during CAPD [2–4]. It has been reported that high glucose suppresses the growth and regeneration of mesothelial cells [5], induces the transforming growth factor-β (TGF-β) gene expression, and increases the oxidant injury and advanced glycosylation products, all of which may contribute to the peritoneal fibrosis [6]. However, the mechanisms are not fully understood.

Macrophages in the peritoneal cavity seem to participate in the process of peritoneal fibrosis through the production of various cytokines and growth factors [7]. Some of these factors induce the cell proliferation and extracellular matrix production in mesothelial cells and fibroblasts, which may lead to peritoneal fibrosis [6, 8–10]. The mechanisms by which monocytes enter the peritoneal cavity are not fully understood, but it is likely that various chemotactic factors and adhesion molecules are involved [11–13]. Monocyte chemoattractant protein-1 (MCP-1), a potent chemokine with considerable specificity for monocytes, has been reported to play a key role in the recruitment of monocytes toward the peritoneal cavity [14]. In addition to chemotactic activity, MCP-1 can induce calcium flux and respiratory burst activity and also regulate the adhesion molecule expressions and cytokine productions in monocytes [15, 16]. It is also known that MCP-1 can stimulate the TGF-β production in lung fibroblast [17].

However, the effects of high glucose on MCP-1 expression in human peritoneal mesothelial cells (HPMCs) are not yet known.

Several signaling pathways have been proposed for the

transcriptional regulation of MCP-1. In the human MCP-1 gene, tissue-type plasminogen activator (t-PA)–responsive elements (TRE) and κB enhancer element exist at the 5′-flanking region of MCP-1 gene, suggesting the role of activator protein-1 (AP-1) and nuclear factor-κB (NF-κB) [18]. In addition, several cellular kinases are involved in the activation of MCP-1 gene, including protein kinase C (PKC) and protein tyrosine kinase (PTK) [19]. Although it has been widely assumed that high glucose increases the expression of NF-κB via the PKC-dependent pathway [20], little has been known about the high-glucose–mediated signal transduction pathways involved in mesothelial MCP-1 gene expression.

The purpose of this study was to evaluate the effect of high glucose on MCP-1 expression in HPMCs and to determine the high-glucose–mediated signal transduction pathways involved in MCP-1 gene expression.

METHODS

Reagent

M199 medium and other cell culture reagents were obtained from GIBCO BRL (Grand Island, NY, USA). The human MCP-1 enzyme-linked immunosorbent assay (ELISA) kit (Quantikine) and anti-human MCP-1 antibody were obtained from R&D Systems Inc. (Minneapolis, MN, USA). The Tri Reagent Kit® was obtained from Molecular Research Center, Inc. (Cincinnati, OH, USA). Moloney murine leukemia virus H-reverse transcriptase (Superscript) was obtained from BRL (Gaithersburg, MD, USA), and other reagents used for reverse transcription were obtained from Boehringer Mannheim (Mannheim, Germany). Genistein and herbimycin A were obtained from Sigma Chemical Company (St. Louis, MO, USA).

Double-stranded AP-1 and NF-κB oligonucleotides were obtained from Promega (Madison, WI, USA).

Cell culture

Human peritoneal mesothelial cells (HPMC) were obtained from omental tissue of consenting patients undergoing elective abdominal surgery. Cells were isolated by modification of a method described previously [21]. Briefly, omental tissue was rinsed in phosphate-buffered saline (PBS), transferred to a solution of 0.125% trypsin and 0.01% ethylenediaminetetraacetic acid (EDTA), and allowed to float for 20 minutes at 37°C. Thereafter, fat tissue was removed, and detached mesothelial cells were collected by centrifugation (5 min at 50 × *g*). HPMCs were cultured in M199 medium supplemented with 10% fetal calf serum, penicillin (100 U/mL), streptomycin (10 μg/mL), and fungizone (0.25 ng/mL) at 37°C in a humidified atmosphere of 5% CO_2. The media were changed every three days, and cells were subcultured by 0.05% trypsin and 0.02% EDTA when they became confluent. HPMCs were identified by typical cobblestone appearance and by the presence of vimentin and cytokeratin and the absence of factor VIII antigen using immunofluorescence methods. Mesothelial cells under passage 3 were used for the experiments.

Cell culture conditioning

To evaluate the effect of chronic exposure of high glucose concentration on the MCP-1 expression, HPMCs were conditioned in 100 mm culture dish with glucose (5 to 100 mmol/L) for varying periods up to seven days in the presence of serum-containing media [22]. Culture media were changed every three days. To examine the effect of osmolality on MCP-1 expression, HPMCs were conditioned with mannitol (50 to 100 mmol/L) for six days.

Monocyte chemoattractant protein-1 mRNA expression, MCP-1 protein expression, and chemotactic activities were measured at the time points indicated.

To examine the role of transcription factor AP-1 and NF-κB in high-glucose–induced MCP-1 gene expression, electrophoretic mobility shift assay (EMSA) was performed at the time points indicated. The effect of AP-1 inhibitor curcumin (7.5 to 15 μmol/L) on the high-glucose–induced MCP-1 mRNA expression and on AP-1 or NF-κB binding activity was examined.

To determine the involvement of tyrosine kinase pathway in glucose-induced MCP-1 expression, HPMCs were preincubated for one hour with PTK inhibitors such as genistein (12.5 to 50 μmol/L) and herbimycin A (0.1 to 1 μmol/L), and then Northern blot analysis and EMSA were performed. Cell viability measured by trypan blue exclusion was not adversely effected by these inhibitors in this experiment.

RNA isolation and Northern hybridization

Total cellular RNA was extracted using a commercially available modification of the acid-phenol method (Tri Reagent Kit®). The amount of RNA was quantitated by absorbance at 260 nm, and purity of RNA was assessed by absorbance ratio at 260/280 nm. Northern hybridization was performed as previously described [23]. Briefly, RNA (10 μg/lane) was electrophoresed through 1% agarose, 2.2 μmol/L formaldehyde denaturing gel with MOPS buffer, followed by capillary transfer to nylon membranes. Filters were stained with methylene blue to check the integrity, uniformity of loading, and transfer of RNA prior to fixation in vacuo at 80°C for two hours. Filters were prehybridized at 65°C in 0.5 mol/L $NaHPO_4$ buffer, pH 7.0, 1 mmol/L EDTA, 7% sodium dodecyl sulfate (SDS), and 1% bovine serum albumin for four to six hours. Then, filters were hybridized with ^{32}P-labeled cDNA probes and 100 μg/mL salmon sperm DNA at 65°C overnight. cDNA probes were labeled with ^{32}P using random primers (Megaprime™ DNA labeling system; Amersham International, Buckinghamshire, UK). Filters were washed and exposed to film at −70°C. Intensit-

ies of the bands on autoradiogram were quantitated by the scanning laser densitometry (GS-670 imaging densitomter; Bio-Rad, Hercules, CA, USA). The same filters were rehybridized with a cDNA specific for glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) to correct for variation in RNA loading and transfer efficiency.

Generation of cDNA probes

cDNA probes specific for human MCP-1 and GAPDH were made by the reverse transcription-polymerase chain reaction (RT-PCR) method. RT-PCR was performed as previously described [23]. Briefly, oligo (dT) (0.5 μg; Boehringer-Mannheim, Mannheim, Germany) was annealed to total RNA (20 μg, from interleukin-1β–stimulated human mesothelial cells) after denaturation at 70°C, and cDNA was synthesized at 42°C for one hour using 100 U of Superscript (BRL, Gaithersburg, MD, USA) in a final volume of 20 μL of the vendor's buffer supplemented with 20 U RNAsin (Promega Biotec, Madison, WI, USA). cDNA was precipitated and dissolved in 1 mmol/L EDTA, 10 mmol/L Tris, pH 7.4. After RT, PCR was performed in PCR buffer containing 0.25 mmol/L dNTP, 12.5 pmol of each primer and 1.25 U of Taq polymerase (Perkin-Elmer Cetus, Norwalk, CT, USA) in a total volume of 50 μL with initial denaturation at 94°C for 3.5 minutes, followed by 30 cycles of 94°C for 1.5 minutes, 54°C for 1.5 minutes, and 72°C for 1.5 minutes. After 30 cycles, a final extension at 72°C for eight minutes was performed. Each PCR primer set was synthesized (Korea Biotech, Inc., Seoul, Korea) according to the published cDNA sequences for human MCP-1 and GAPDH [24, 25].

The sequences of each primer set were as follows: MCP-1, forward primer 5′-AAGCTCGCACTCTCGC CTC-3′ and backward primer 5′-CAGGGTGTCTGGG GAAAGC-3′; GAPDH, forward primer 5′-TCACCAT CTTCCAGGAGCG-3′ and backward primer 5′-CTGC TTCACCACCTTCTTGA-3′.

Polymerase chain reaction products were electrophoresed on an ethidium bromide-stained 1% agarose gel, and the expected size of each PCR product was confirmed under ultraviolet illumination. PCR products were purified using a Jetsorb gel extraction kit (Genomed, Inc., Research Triangle Park, NC, USA).

Enzyme-linked immunosorbent assay for MCP-1 protein

Monocyte chemoattractant protein-1 protein in cell culture supernatant was measured by enzyme-linked immunosorbent assay (ELISA). The ELISA method was a specific sandwich enzyme immunoassay (Human MCP-1 kit, Quantikine; R&D). Briefly, diluted cell culture supernatant was added to 96-well microtiter plate that was coated with murine monoclonal antibody against human MCP-1. After incubation at room temperature for two hours and careful washes, polyclonal antibody conjugated to horseradish peroxidase against MCP-1 was added. After another incubation for one hour at room temperature and repeated washes, color reagents were added. Optical density of each well was measured at 450 nm with an ELISA reader, using a 570 nm reference wavelength.

Chemotaxis assay

The chemotactic activity of conditioned cell culture supernatant was assessed using 48-well microchemotaxis chambers (Neuroprobe, Cabin Jhon, MD, USA) as previously described [26]. Briefly, human peripheral blood mononuclear cells were isolated by centrifugation through Ficoll-Hypaque. Monocytes were purified from these preparations by the method of Rich et al [27]. Cell viability was evaluated by trypan blue exclusion and should be >95%. Purified monocytes suspensions (5×10^4 cells/well) were added to the top well of the chamber and permitted to migrate through a 10 μm thick, 5 μm porosity polycarbonate membrane (Nucleopore Corp., Pleasanton, CA, USA) toward the sample-bearing bottom chamber, which contained conditioned cell culture supernatant. Monocytes were incubated for 90 minutes at 37°C in humidified air with 5% CO_2. After incubation, the membrane was removed. Nonmigrating cells were wiped off, and the polycarbonate membrane was fixed for five minutes in absolute methanol, followed by air drying and staining for 30 minutes in Wright's Giemsa. The number of cells migrating through the membrane was counted in three random 10 mm grids at ×400 for triplicated samples. Chemotactic activity was standardized to and expressed as the percentage of the positive control (10^{-8} mol/L, f-Met-Leu-Phe) as previously reported [26, 28, 29].

To examine whether glucose-induced chemotactic activity for monocytes was mediated through MCP-1, a neutralizing test was performed using specific anti–MCP-1 antibody (20 μg/mL) and an equivalent concentration of nonspecific antibody.

Extraction of nuclear protein and EMSA

Nuclear extracts were prepared by modification of a method described previously [30]. Briefly, cells were gently washed twice with ice-cold PBS and scraped in 1 mL of ice-cold hypotonic lysis buffer [10 mmol/L HEPES-KOH, pH 7.9, 60 mmol/L KCl, 1 mmol/L EDTA, 1 mmol/L dithiothreitol (DTT), 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), and 0.5% Nonidet P-40]. The cells were vortexed and allowed to swell on ice for 10 minutes. The nuclei were collected by centrifugation at 2000 r.p.m. for five minutes at 4°C. The nuclear pellet were resuspended in 100 μL of high salt extraction buffer (20 mmol/L HEPES-KOH, pH 7.9, 0.42 mol/L NaCl, 1.2 mmol/L $MgCl_2$, 0.5 mmol/L DTT, 0.2 mmol/L EDTA, 25% glyc-

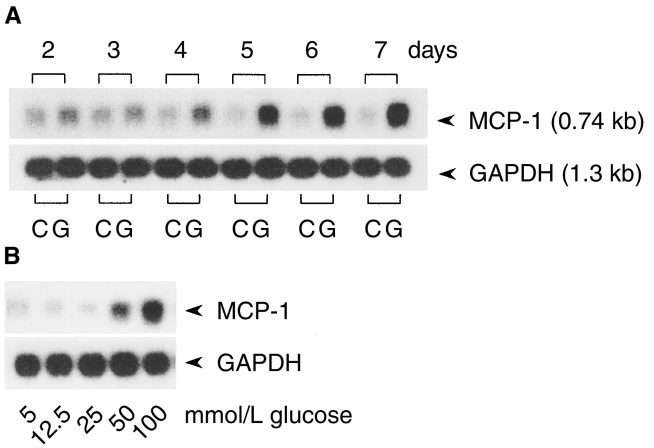

Fig. 1. Induction of monocyte chemoattractant protein-1 (MCP-1) mRNA by glucose. (*A*) Time-dependent induction of MCP-1 mRNA by glucose. Human peritoneal mesangial cells (HPMCs) were exposed to 5 mmol/L glucose [control (C)] and 100 mmol/L glucose (G) for up to seven days. Northern blot analysis was performed. Results shown are representative of three experiments. (*B*) Dose-dependent induction of MCP-1 mRNA by glucose: HPMCs were exposed to varying concentration of glucose for five days.

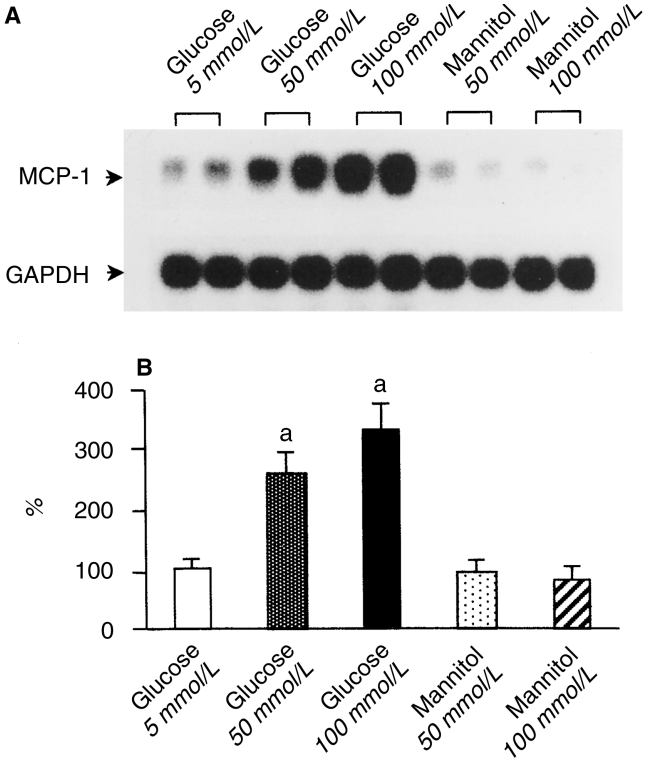

Fig. 2. Quantitation of MCP-1 mRNA expression in mesothelial cells cultured for six days with glucose or mannitol. HPMCs were exposed to 5 mmol/L glucose (control), 50 mmol/L, 100 mmol/L glucose, 50 mmol/L mannitol, or 100 mmol/L mannitol for six days. Northern blot analysis was performed (*A*), and the MCP-1 mRNA level was normalized by the GAPDH mRNA level (*B*). Data were mean ± SE of three experiments. $^{a}P < 0.05$ vs. control.

erol, 0.5 mmol/L PMSF, 5 μg/mL aprotinin, 5 μg/mL leupeptin) and incubated for 20 minutes on ice with shaking. The nuclear extract was then centrifuged for 15 minutes at 4°C, and the supernatant was stored at −80°C. Protein concentration was determined using a colorimetric assay based on the method of Bradford [31].

Electrophoretic mobility shift assay was performed by incubation of nuclear protein (10 μg) with ^{32}P-labeled AP-1 or NF-κB oligonucleotides at room temperature for 20 minutes in binding buffer containing 50 mmol/L Tris, pH 7.5, 250 mmol/L NaCl, 20% glycerol, 5 mmol/L $MgCl_2$, 2.5 mmol/L EDTA, 2.5 mmol/L DTT, 0.25 mg/mL poly (dI-dC). Double-stranded AP-1 oligonucleotides (5′-CGC TTGATGAGTCAGCCGGAA-3′) and NF-κB oligonucleotides (5′-AGTTGAGGGGACTTTCCCAGGC-3′) were labeled with γ-^{32}P-ATP using T4 kinase and purified on a Sephadex G-25 column. Nucleoprotein-oligonucleotide complexes were resolved by electrophoresis on 4% nondenaturing polyacrylamide gels in 0.25 × TBE buffer. The gel was dried and autoradiographed with intensifying screen at −70°C. Competition assay was performed by 10 minutes of preincubation of nuclear protein with 100 times excess unlabeled AP-1 or NF-κB oligonucleotides and nonspecific oligonucleotides

Statistics

All data were expressed as mean ± SE. Groups were compared using an analysis of variance test for multiple group comparison (Scheffe's method). A *P* value less than 0.05 was considered significant.

RESULTS

Effect of high glucose and mannitol on MCP-1 mRNA expression

Incubation of HPMCs with high glucose (100 mmol/L) increased the MCP-1 mRNA expression in a time-dependent (2 to 7 days) and dose-dependent manner (12.5 to 100 mmol/L glucose; Fig. 1). HPMCs cultured in 50 mmol/L and 100 mmol/L glucose for six days demonstrated an increase in MCP-1 mRNA expression approximately 2.6 ± 0.3-fold and 3.3 ± 0.4-fold, respectively, when compared with cells cultured in 5 mmol/L glucose (Fig. 2). To examine the effect of osmolality on MCP-1 mRNA expression, HPMCs were cultured in 50 and 100 mmol/L mannitol for six days. As shown in Figure 2, incubation of HPMCs with 50 and 100 mmol/L mannitol had no significant effect on MCP-1 mRNA expression.

Effect of high glucose and mannitol on MCP-1 protein expression

Monocyte chemoattractant protein-1 protein levels in cell culture supernatant were determined from HPMCs

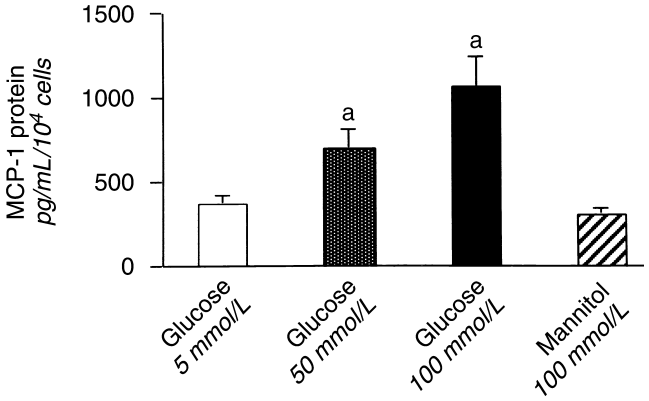

Fig. 3. Effect of glucose and mannitol on the cell supernatant MCP-1 protein. HPMCs were exposed to 5 mmol/L glucose (control), 50 mmol/L glucose, 100 mmol/L glucose, and 100 mmol/L mannitol for six days. MCP-1 protein in cell culture supernatant was measured by ELISA. [a]$P < 0.05$ vs. control.

cultured in 5, 50, 100 mmol/L glucose and 100 mmol/L mannitol for six days. As shown in Figure 3, incubation of HPMCs with 50 and 100 mmol/L glucose for six days increased the MCP-1 protein level approximately 1.9 ± 0.2- and 2.8 ± 0.4-fold, respectively, when compared with cells cultured in 5 mmol/L glucose; 100 mmol/L mannitol had no significant effect. Each data point in Figure 3 represented the MCP-1 protein level for the previous 72 hours because all cell culture media were changed every 72 hours.

Effect of high glucose on the chemotactic activity for monocytes

Chemotactic activity was standardized to and expressed as the percentage of the positive control (10^{-8} mol/L, f-Met-Leu-Phe) as previously reported [26, 28, 29].

Supernatants obtained from HPMCs cultured in 100 mmol/L glucose for six days showed higher levels of chemotactic activity for monocytes than those from HPMCs cultured in 5 mmol/L glucose and media containing 100 mmol/L glucose only (Fig. 4).

To examine whether high-glucose–induced chemotactic activity was mediated through MCP-1, we attempted to neutralize the chemotactic activity of the conditioned supernatant with a specific anti–MCP-1 antibody. The addition of a specific anti–MCP-1 antibody (20 μg/mL) significantly inhibited the chemotactic activity induced by high glucose. Equivalent concentration of nonspecific antibody did not affect the chemotactic activity induced by high glucose. Furthermore, f-Met-Leu-Phe (10^{-8} mol/L)-induced chemotactic activity was not altered by anti–MCP-1 antibody (Fig. 4). These findings suggested that high-glucose–induced MCP-1 production contributed to a significant proportion of the overall high-glucose–induced monocyte chemotactic activity.

In addition, basal chemotactic activity from HPMCs cultured in 5 mmol/L glucose was also inhibited by anti–MCP-1 antibody (Fig. 4).

Involvement of transcription factor AP-1 in glucose-induced MCP-1 gene expression

It has been reported that activation of AP-1 or NF-κB is essential for the induction of MCP-1. To examine whether high-glucose–induced MCP-1 gene expression was mediated by the activation of AP-1 or NF-κB, nuclear protein extracts from mesothelial cells stimulated by high glucose were subjected to EMSA. Because Northern blot analysis revealed that high glucose increased MCP-1 mRNA expression in a time-dependent (2 to 7 days) manner, EMSAs were performed with nuclear proteins obtained from mesothelial cells cultured for one, three, and five days in 5 and 100 mmol/L glucose, respectively. EMSA revealed that high glucose increased the AP-1 binding activity in a time-dependent (1 to 5 days) and dose-dependent manner (12.5 to 100 mmol/L glucose; Fig. 5). However, there were no significant changes in the activity of NF-κB binding (Fig. 6A). A competition assay was performed to demonstrate specificity of AP-1 and NF-κB binding activity observed in the EMSA. Preincubation with excess unlabeled double-stranded AP-1 or NF-κB oligonucleotides in the DNA binding reaction effectively blocked the formation of specific DNA/protein complex. Nonspecific oligonucleotides did not affect the AP-1 and NF-κB binding activity observed in the EMSA (Figs. 5C and 6B).

The effects of a pharmacological inhibitor of AP-1, curcumin, on the high-glucose–induced MCP-1 mRNA expression and high-glucose–induced AP-1 binding activity was examined further. Curcumin dose-dependently suppressed the high-glucose–induced MCP-1 mRNA expression (Fig. 7) and the high-glucose–induced AP-1 activity (Fig. 8B). However, curcumin inhibited the basal NF-κB binding activity both in control cells and in cells stimulated with glucose (Fig. 9).

Involvement of PTK in high glucose-induced MCP-1 gene expression

Several cellular kinases are involved in the activation of MCP-1 gene, including PTK. To determine whether the activation of tyrosine kinase was an essential event for the MCP-1 induction by glucose, we examined the effect of PTK inhibitor such as genistein and herbimycin A on the high glucose-induced MCP-1 mRNA expression. Northern blot analysis revealed that genistein (12.5 to 50 μmol/L) and herbimycin (0.1 to 1 μmol/L) suppressed the high-glucose–induced MCP-1 mRNA expression in a dose-dependent manner (Fig. 8A).

To investigate whether the activation of tyrosine kinase was located upstream of AP-1, we examined the effect of tyrosine kinase inhibitors on high-glucose–induced

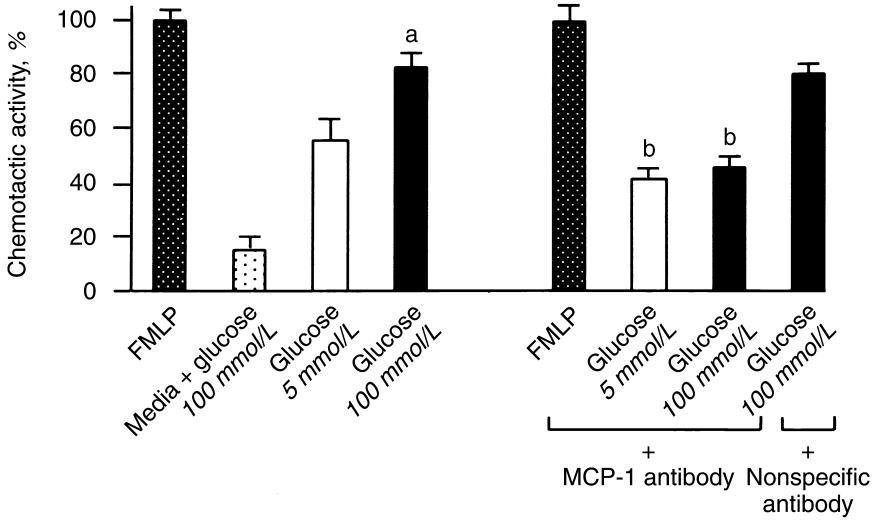

Fig. 4. Effect of high glucose on the chemotactic activity for monocytes and inhibition by anti–MCP-1 antibody. HPMCs were exposed to 5 mmol/L glucose (control) and 100 mmol/L glucose for six days. Chemotactic activity in cell-free supernatants before and after the addition of anti–MCP-1 antibody (20 μg/mL) or nonspecific antibody (20 μg/mL) was measured by chemotactic assay. Data are expressed as a percentage of maximum monocyte chemotactic response to f-Met-Leu-Phe (FMLP) 10^{-8} mol/L. Results were expressed as mean ± SE of six experiments. [a]$P < 0.05$ vs. control; [b]$P < 0.05$ vs. before the addition of anti–MCP-1 antibody.

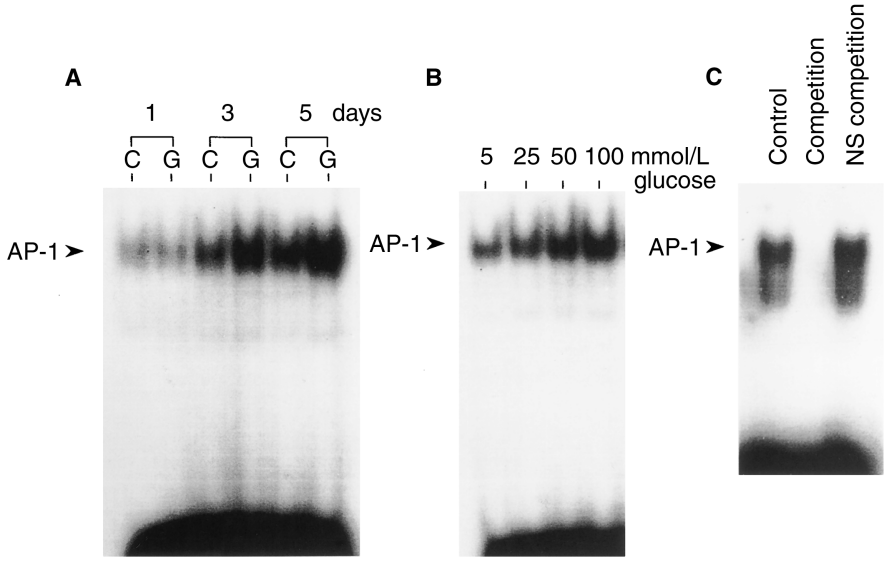

Fig. 5. Effect of high glucose on activator protein-1 (AP-1) binding activity. (*A*) Time-dependent increase in AP-1 binding activity by high glucose. Electrophoretic mobility shift assays (EMSA) were performed with nuclear proteins from HPMCs cultured for up to five days in 5 mmol/L glucose (C) and 100 mmol/L glucose (G). Results shown are representative of three experiments. (*B*) Dose-dependent increase in AP-1 binding activity by glucose. EMSAs were performed from mesothelial cells cultured with varying doses of glucose for three days. (*C*) Competition assay of AP-1 binding activity. Competition assay was performed to demonstrate the specificity of AP-1 binding activity. Nuclear protein from HPMCs cultured for three days in 100 mmol/L glucose (Control) was preincubated with 100 times excess unlabeled AP-1 oligonucleotide (Competition) and nonspecific oligonucleotide (NS competition) for 10 minutes, and then EMSA was performed.

AP-1 binding activity. EMSA revealed that genistein and herbimycin suppressed the high-glucose–induced AP-1 binding activity at the concentration that inhibited the high-glucose–induced MCP-1 mRNA expression (Fig. 8B).

We further examined the effect of tyrosine kinase inhibitors on NF-κB binding activity, and found that they had no significant effect (Fig. 9A).

These findings suggested that tyrosine kinase was required for the activation of AP-1, which was essential to the induction of MCP-1 gene expression by glucose.

DISCUSSION

This study simulated the high-glucose conditions in CAPD by using mesothelial cells cultured under high-glucose concentrations of up to 100 mmol/L for up to seven days. Then, the effect of high glucose on the MCP-1 expression in mesothelial cells was evaluated. Next, we investigated the high-glucose–mediated signal transduction pathways involved in MCP-1 gene expression.

Mesothelial cells are the most abundant cells in the peritoneum and play an important role in the control of inflammation in the peritoneal cavity. Induction of MCP-1 in mesothelial cells by various cytokines and growth factors, including interleukin-1β, tumor necrosis factor-α, and interferon-γ, has been demonstrated [32]. MCP-1 has been reported to play a key role in the recruitment of monocytes toward the peritoneal cavity [14]. In addition to its role in cellular immune reactions and responses to acute tissue injury, MCP-1 has been suggested as an important mediator in human diseases. This is supported by evidence that MCP-1 is expressed in

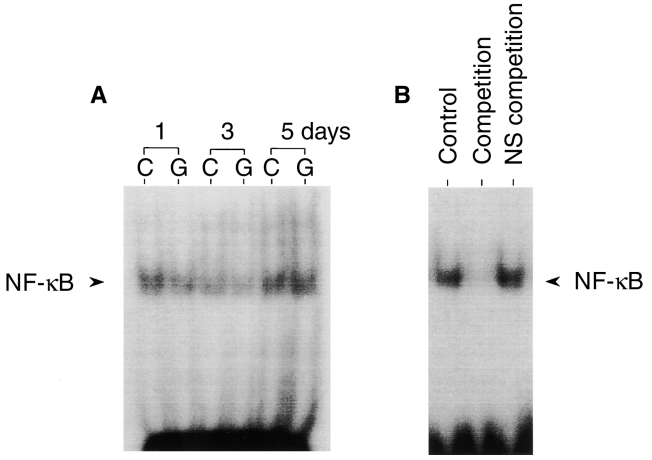

Fig. 6. Effect of high glucose on nuclear factor-κB (NF-κB) binding activity. (*A*) EMSAs were performed with nuclear proteins from HPMCs cultured for up to five days in 5 mmol/L glucose (C) and 100 mmol/L glucose (G). Results shown are representative of three experiments. (*B*) Competition assay was performed to demonstrate the specificity of NF-κB binding activity. Nuclear protein from HPMCs cultured for three days in 100 mmol/L glucose (Control) was preincubated with 100 times excess unlabeled NF-κB oligonucleotide (Competition) and nonspecific oligonucleotide (NS competition) for 10 minutes, and then EMSA was performed.

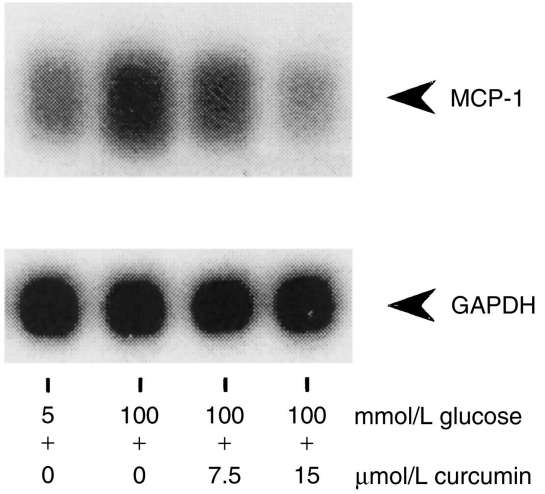

Fig. 7. Effect of AP-1 inhibitor, curcumin, on induction of MCP-1 mRNA by high glucose. HPMCs grown in normal (5 mmol/L) and high glucose (100 mmol/L) for three days were treated with curcumin for 24 hours, and then Northern blot analysis was performed.

atherosclerotic lesion [33] and glomerulonephritis [34]. Similarly, MCP-1 is expressed in human melanoma but not in normal skin [35].

However, little has been known about the effect of high glucose on MCP-1 expression in HPMCs.

We found that glucose increased MCP-1 mRNA expression in a time- (2 to 7 days) and dose-dependent manner. MCP-1 protein in cell culture supernatant was also increased. An equivalent concentration of mannitol had no significant effect on the MCP-1 mRNA and protein expression, suggesting that glucose-induced up-regulation of MCP-1 was independent of osmolality. Furthermore, high-glucose–conditioned supernatant possessed the increased chemotactic activity for monocytes, which was neutralized by specific anti–MCP-1 antibody. The addition of anti–MCP-1 antibody almost completely inhibited the increased portion of chemotactic activity by high glucose, suggesting that increased portion of chemotactic activity by high glucose was mainly due to MCP-1 production by high glucose. These findings may suggest a possibility that high glucose in peritoneal dialysis solutions may contribute to the peritoneal fibrosis by up-regulating the mesothelial synthesis of MCP-1. In addition, long-term exposure of high glucose in the peritoneal dialysate of CAPD patients has been shown to facilitate advanced glycation end product (AGE) formation in the peritoneal membrane [36], and an accumulation of AGEs induces the progression of peritoneal fibrosis and microvascular sclerosis [37].

Basal chemotactic activity from control cells cultured in 5 mmol/L glucose in our study appeared higher than expected. We thought that one of the possible reasons was that, in our study, cells were conditioned with glucose in the presence of serum-containing media.

Monocyte chemoattractant protein-1 gene can be activated by PKC- or PTK-dependent pathway and downstream transcription factors such as AP-1 or NF-κB [19]. It has been widely assumed that high glucose increases the expression of NF-κB and that this process is mediated via PKC-dependent pathway [20], which suggests a possible role of the PKC–NF-κB pathway in high-glucose–induced MCP-1 gene expression. However, another important aspect of MCP-1 signal transduction is the transcription activation promoted by the binding of AP-1 to TRE. Previous reports have shown that activation of AP-1 is required for the induction of MCP-1 by lipopolysaccharide, mechanical stress, TGF-β in macrophages, vascular endothelial cells, and osteoblastic cells, respectively [38–40]. Furthermore, increased binding of AP-1 to TRE in high-glucose environments was also reported in human mesangial cells cultured chronically for five days [22] and in porcine vascular smooth muscle cells cultured chronically for three to nine days [41], suggesting a possible role of AP-1 in high-glucose–induced MCP-1 gene expression.

To examine whether high-glucose–induced MCP-1 gene expression was mediated by the activation of AP-1 or NF-κB, we measured the activity of AP-1 and NF-κB in mesothelial cells cultured with high glucose for one, three, and five days because high glucose increased MCP-1 mRNA expression progressively during the study periods (2 to 7 days).

We found that high glucose increased the AP-1 bind-

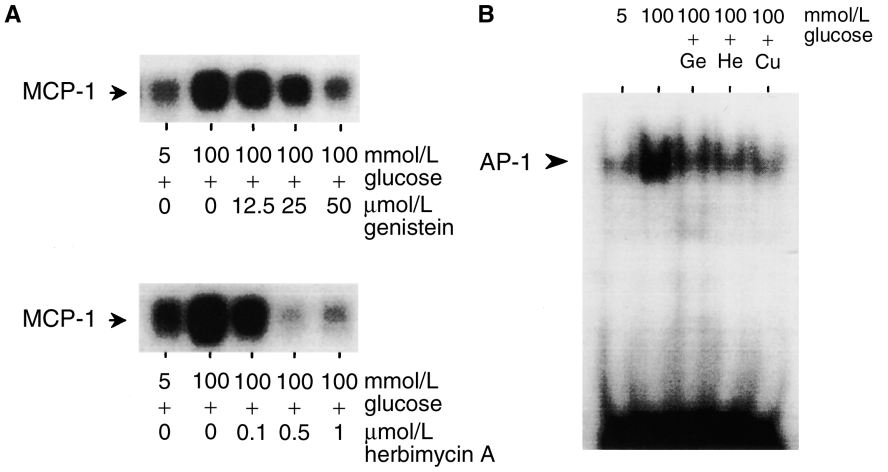

Fig. 8. Effect of tyrosine kinase inhibitors on high-glucose–induced MCP-1 mRNA expression and high-glucose–induced AP-1 binding activity. (*A*) HPMCs were exposed to 100 mmol/L glucose for three days with or without varying dose of genistein and herbimycin A. Northern blot analysis was performed. Results shown are representative of two experiments. (*B*) Suppression of high-glucose–induced AP-1 binding activity by genistein, herbimycin A and curcumin. EMSAs were performed with nuclear proteins from mesothelial cells cultured for three days in 100 mmol/L glucose with or without 50 μmol/L genistein (Ge), 1 μmol/L herbimycin A (He), and 15 μmol/L curcumin (Cu). Results shown are representative of three experiments.

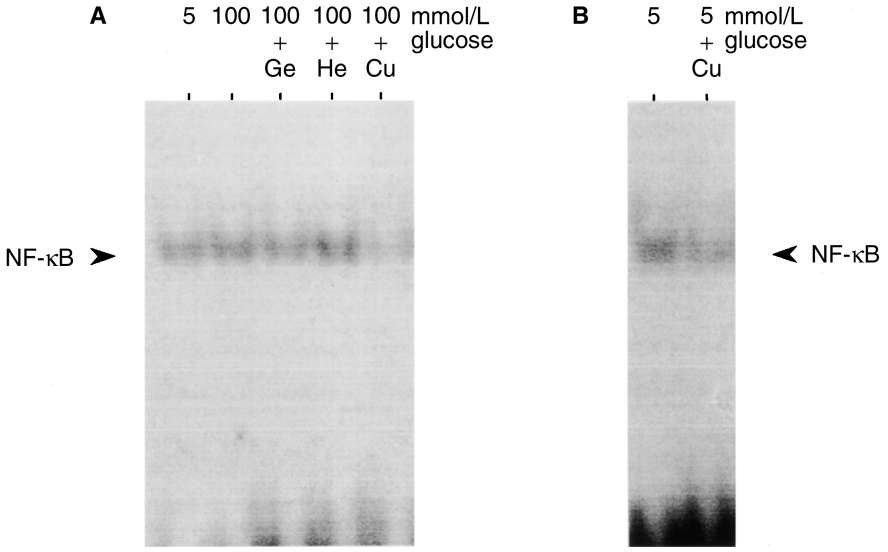

Fig. 9. Effect of tyrosine kinase inhibitors and curcumin on the NF-κB binding activity. (*A*) Effect of genistein (Ge), herbimycin A (He), and curcumin (Cu) on the NF-κB binding activity in mesothelial cells incubated with 100 mmol/L glucose. EMSAs were performed with nuclear proteins from mesothelial cells cultured for three days in 100 mmol/L glucose with or without 50 μmol/L genistein (Ge), 1 μmol/L herbimycin A (He), and 15 μmol/L curcumin (Cu). Results shown are representative of three experiments. (*B*) EMSAs were performed with nuclear proteins from mesothelial cells cultured for three days in 5 mmol/L glucose (control) with or without 15 μmol/L curcumin (Cu).

ing activity in a time- and dose-dependent manner. Furthermore, a pharmacological inhibitor of AP-1, curcumin, dose-dependently suppressed the high-glucose–induced MCP-1 mRNA expression and the high glucose-induced AP-1 activity. These findings suggest that activation of AP-1 is essential to induction of MCP-1 gene expression in mesothelial cells cultured chronically with high glucose.

However, curcumin has been reported to inhibit NF-κB activity as well [42]. Therefore, we examined the effect of curcumin on the NF-κB binding activity. In our study, curcumin suppressed the basal NF-κB binding activity both in control cells and in cells stimulated with glucose.

It has been widely assumed that high glucose increases the expression of NF-κB [20]. However, we found no significant changes in NF-κB binding activity in mesothelial cells cultured with high glucose for one, three, and five days. This discrepancy may be explained by the fact that the increase in high-glucose–induced NF-κB activity is a relatively early event. In endothelial cells, high-glucose–induced NF-κB activity was observed as early as one hour after stimulation (peak activation at 2 to 4 hours) and then decreased thereafter [43].

Several cellular kinases are involved in the activation of the MCP-1 gene, including PKC and PTK [19]. Previous studies have shown that activation of PTK is required for the induction of MCP-1 gene expression by PDGF, IL-1β and TNF-α in renal mesangial cells [44–46]. To determine whether the activation of PTK was an essential event for the glucose-induced MCP-1 gene expression, we examined the effect of PTK inhibitors such as genistein and herbimycin A on high-glucose–induced MCP-1 gene expression. We found that both genistein and herbimycin A suppressed the high-glucose–induced MCP-1 mRNA expression in a dose-dependent manner. Furthermore, genistein and herbimycin A suppressed the high-glucose–induced increased AP-1 binding activ-

ity but not NF-κB, suggesting that MCP-1 expression, especially in mesothelial cells cultured chronically with high glucose, was mediated at least in part via the tyrosine kinase–AP-1 pathway.

In summary, chronic exposure of high glucose concentration for up to seven days increases the MCP-1 expression in HPMCs. MCP-1 secreted into culture media enhances monocyte migration. Tyrosine kinase and AP-1 are involved in the induction of MCP-1 by high glucose. These results suggest that high glucose induces MCP-1 expression in peritoneal mesothelial cells partly via the tyrosine kinase–AP-1 pathway.

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APPENDIX

Abbreviations used in this article are: AGE, advanced glycation end product; AP-1, activator protein-1; CAPD, continuous ambulatory peritoneal dialysis; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme-linked immunosorbent assay; EMSA, electrophoretic mobility shift assay; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HPMC, human peritoneal mesothelial cells; IFN-γ, interferon-γ; IL-1β, interleukin-β; MCP-1, monocyte chemoattractant protein-1; NF-κB, nuclear factor-κB; PBS, phosphate-buffered saline; PDGF, platelet-derived growth factor; PKC, protein kinase C; PMSF, phenylmethylsulfonyl fluoride; PTK, protein tyrosine kinase; RT-PCR, reverse transcription-polymerase chain reaction; SDS, sodium dodecyl sulfate; TGF-β, transforming growth factor-β; TRA, 12-*O*-tetradecanoylphorbol 13-acetate; TRE, TRA-responsive element.

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